ab65332

Hemin Assay Kit

Instructions for Use

For the rapid, sensitive and accurate measurement of

Hemin levels in various samples

View kit datasheet: www.abcam.com/ab65332

(use www.abcam.cn/ab65332 for China, or www.abcam.co.jp/ab65332 for Japan)

This product is for research use only and is not intended for diagnostic use.

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

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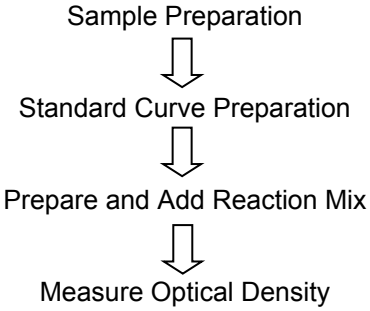
1. Overview

Free hemin results from the breakdown of hemin-containing proteins such as hemoglobin and myoglobin. It can be detected in various body fluids such as saliva, urine and CSF under various pathological states. Free hemin exists in cells at very minute concentrations (<1μM ≈ 650 ng/ml) exerting regulatory functions such as repression of nonspecific δ-aminolevulinate synthase expression and induction of microsomal hemin oxygenase-1. Hemin can stimulate growth of oral bacteria involved in gingivitis and is an indicator of possible pathological conditions when found in the urine or feces.

Abcam's Hemin Assay Kit utilizes peroxidase activity in the presence of hemin to provide a simple, exquisitely sensitive assay which causes the conversion of a colorless probe to a strongly colored (λ = 570) compound. Trace amounts of Hemin can be quantitated in the 5-160 pg (10-250 fmol) range.

2. Protocol Summary



3. Components and Storage

A. Kit Components

Item	Quantity
Assay Buffer V/Hemin Assay Buffer	25 mL
OxiRed Probe II/Hemin Probe (in DMSO)	0.2 mL
Apoperoxidase Enzyme Mix/Enzyme Mix (Lyophilized)	1 vial
Substrate V/Hemin Substrate	1 mL
Hemin Standard (1 nmol; Lyophilized)	1 vial

* Store kit at -4°C.

OxiRed Probe II/PROBE: Warm at 37°C for 1-2 min to completely melt before use. Mix well, store at 4°C, protect from light and moisture. Use within two months.
Apoperoxidase Enzyme Mix/ENZYME MIX: Dissolve in 0.5 ml Assay Buffer V/Hemin Assay Buffer, mix well. Store at -20°C.
Substrate V/HEMIN SUBSTRATE: Ready to use as supplied. Store at 4°C. Use within two months.

HEMIN Standard: Dissolve with 100 µl DMSO to make a 10 µM solution. Store at 4°C. Use within two months

B. Additional Materials Required

- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader
- 96 well plate
- Orbital shaker

4. Assay Protocol

1. Sample Preparation:

Depending upon the hemin content, samples should be diluted typically 100 to 10,000-fold and added at about 1-10 µl of diluted sample per well. Samples can be assayed without any prior treatment.

Hemin concentration in samples may have a wide range. For different sample types, we suggest using ~0.04 µl serum sample, ~50 µg of feces, ~1-5000 cultured cells or ~0.05 µl urine. Place diluted samples directly in wells and adjust well volumes to 50 µl with Assay Buffer V/Hemin Assay Buffer in a 96-well plate.

We suggest using several doses of your sample to ensure the readings are within the standard curve range.

Note:

The presence of hemoproteins may interfere with the assay although in our experience, the very high dilution factor reduces the concentration of any such proteins to undetectable levels.

You may do a sample background control without the Apoperoxidase Enzyme Mix/Enzyme Mix in the reaction, and then subtract the sample background from your sample readings.

As a Quick Guide as to how you may prepare your Tissue Samples.

The Assay Buffer V/Assay buffer contains detergent and can be used to homogenize minced tissue samples (10-100mg) in a Dounce homogenizer Typically 3-4 volumes of the buffer is enough to homogenize tissue. Tissue quick-frozen in liquid nitrogen can be used after grinding and homogenizing in the Assay Buffer V/assay buffer. The homogenate should be spun down to remove debris and then the supernatant can be used for the assay. The amount of tissue depends on the heme content (liver has plenty of heme whereas muscles not so much. Since some tissues have a lot of heme, the samples might need to be diluted to make sure that the hemoproteins do not interfere in the free heme assay.

2. Standard Curve Preparation:

Immediately before use, dilute the 10 µM Hemin Standard to 100 nM by adding 10 µl of the Standard to 990 µl of Assay Buffer V/Hemin Assay Buffer, mix well. Dilute further to 10 nM (= 10 fmol/µl) by adding 100 µl to 900 µl Assay Buffer V/Hemin Assay buffer. Add 0, 4, 8, 12, 16, 20 µl into a series of wells. Adjust volume to 50 µl/well with Assay Buffer V/Hemin Assay Buffer to generate 0, 40, 80, 120, 160, 200 fmol/well of the Hemin Standard.

3. Reaction Mix:

NOTE: The proper order of addition of the following components is critical. Immediately before use, mix enough reagent for the

number of assays performed. For each well, prepare a 50 µl Reaction Mix containing the following components in the following order.

1. Apoperoxidase Enzyme Mix/Enzyme Mix 3 µl
2. Substrate V/Hemin Substrate 2 µl

Incubate for 2 minutes at room temperature before adding the following components:

3. Assay Buffer V/Assay Buffer 43 µl
4. OxiRed Probe II/Probe 2 µl

Incubate the reaction for 10-30 minutes at room temperature, protect from light.

Note:

As this is an enzyme activity assay it is important to incubate and measure your samples at the same time and under exactly the same conditions as the standards.

The 10–30-minute incubation time has been selected as the best compromise between linearity, speed and sensitivity. It is advantageous to read the assay in kinetic mode (as shown below), observing the color development as it proceeds, using measurement data in the range of 0.7-1.3 OD for the highest standard (200 fmol).

4. Measurement: Read the OD at 570 nm.

5. Data Analysis

Correct background by subtracting the value derived from the zero Hemin control from all sample and standard readings. The background reading may be significant and must be subtracted from sample readings.

Plot the standard curve as pmol/well vs. OD 570 nm readings. Then apply the sample readings to the standard curve to get Hemin amount in the sample wells (Hy).

Calculate the Hemin concentrations in the test samples as follows:

Concentration = Hy / Sv x Ds (fmol/µl or nM)

Where:

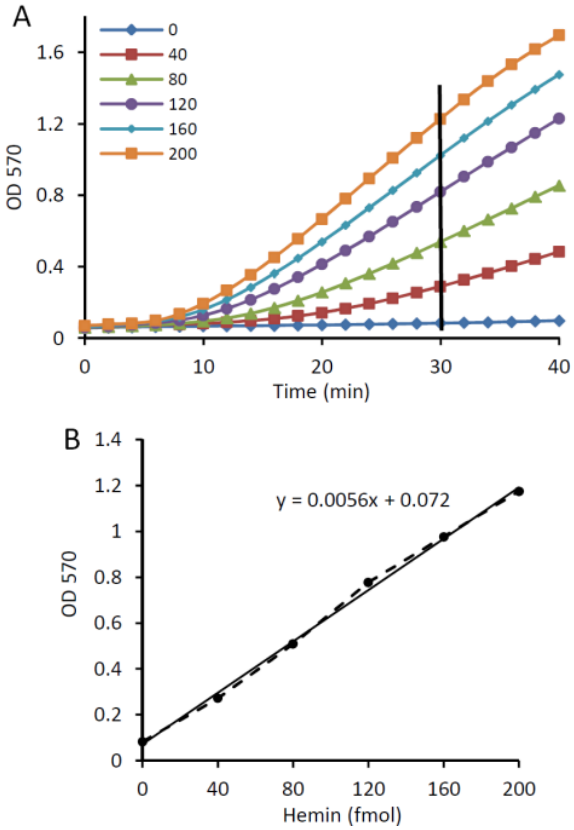
Hy is the amount of Hemin (fmol) of your sample from standard curve.

Sv is the sample volume (µl) added into the sample well.

Ds is the dilution factor of the sample, i.e. 100 or 10,000.

Hemin molecular weight: 652.

Hemin concentration in your sample can be expressed as pmol/ml, ng/ml, µg/dL or µM (µmol/liter); 1 µM = 1 nmol/ml = 652 ng/ml.



(A) Time course of development of color and (B) Standard Curve at 30 minutes for Hemin standard as performed according to this protocol.

6. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay Buffer V/Assay buffer at wrong temperature	Assay Buffer V/Assay buffer must not be chilled - needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Use appropriate reader and filter settings described in datasheet
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Unsuitable sample type	Use recommended samples types as listed on the datasheet
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Samples prepared in the wrong buffer	Use the Assay Buffer V/assay buffer provided (or refer to datasheet for instructions)
	Samples not deproteinized (if indicated on datasheet)	Use the ***10kDa spin column (ab93349)***
	Cell/ tissue samples not sufficiently homogenized	Increase sonication time/ number of strokes with the Dounce homogenizer
	Too many freeze-thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
Lower/ Higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Out-of-date kit or incorrectly stored reagents	Always check expiry date and store kit components as recommended on the datasheet
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect incubation time/ temperature	Refer to datasheet for recommended incubation time and/ or temperature
	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)

Problem	Reason	Solution
Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when preparing the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Concentration of standard stock incorrect	Recheck datasheet for recommended concentrations of standard stocks
	Errors in standard curve calculations	Refer to datasheet and re-check the calculations
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

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